Faculty of Science

THE IMPACT AND MECHANISM OF INTERACTION OF EXERCISE-DERIVED MICROVESICLES ON CULTURED ENDOTHELIAL CELLS

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THE IMPACT AND MECHANISM OF INTERACTION OF EXERCISE-DERIVED MICROVESICLES ON CULTURED ENDOTHELIAL CELLS

By

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ABSTRACT

Microvesicles (MV) were once thought to be inert cellular fragments; however, a large body of research suggests they are bioactive components involved in intercellular communication and altering physiological processes. As these 100 nm – 1 μm plasma membrane-derived vesicles are formed from many cell types, they vary significantly in composition and function. Recently, it has been shown that high intensity exercise increases the concentration of circulating platelet-derived MVs. While the physiological role of these MVs is unknown, they have been shown to improve the functionality of endothelial cells, indicating a potential for mediating the adaptive effects of exercise on the cardiovascular system. The present study aims to further examine the effects of these exercise-derived MVs on the endothelium, as well as investigate the mechanism by which MVs and endothelial cells interact. Exercise trials were performed to produce blood MV samples from eleven participants. To test the effects of exercise-derived MVs, cultured endothelial cells were treated with MV samples from rest, exercise or recovery. Endothelial cell function, in terms of proliferation and wound-healing, was examined. To gain insight into the mechanism of interaction between exercise-derived MVs and endothelial cells, blockade studies were incorporated into the functional assays. MV surface proteins were degraded with trypsin and the interaction between Axl and Gas6 was inhibited with an anti-Axl antibody. Several mistakes were made within the course of this study, including incorrect preparation of the sodium citrate anticoagulant used for blood sample collection and possible microbial contamination of endothelial cell cultures. As such, the results of this study are inconclusive and further research is required to determine the effect of exercise-derived MVs on endothelial cell function and the pathways involved in this interaction.

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LIST OF ABBREVIATIONS

ADP	Adenosine triphosphate
BMI	Body mass index
CAC	Circulating angiogenic cell
CVD	Cardiovascular disease
DAG	Diacylglycerol
Del-1	Developmental endothelial locus 1
EV	Extracellular vesicle
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intracellular adhesion molecule-1
IP_3	Inositol 1,4,5-trisphosphate
MV	Microvesicle
PAH	Pulmonary arterial hypertension
PAK	P21-activated kinase
PDEGF	Platelet-derived endothelial cell growth factor
PIP_3	Phosphatidylinositol 4,5-bisphosphate
PLC	Phospholipase C-β
PMV	Platelet microvesicle
PROSPR	Protein organic solvent precipitation
RPE	Rate of perceived exertion
TAM	Tyro3, Axl and Mer
tMV	Trypsin-treated microvesicle
TRAP	Thrombin receptor-activating
VEGF	Vascular endothelial growth factor

1 LITERATURE REVIEW

1.1 The discovery of microvesicles

In Darwin's book, "The Variation of Plants and Animals under Domestication" (1868), he proposed that cells "throw off minute granules" that circulate throughout the body.[1] Darwin named these cellular granules "gemmules" and thought that they were involved in the incorrectly hypothesized inheritance of acquired characteristics. Of course, Darwin was mistaken about the function of these gemmules, but he had nonetheless foreshadowed the discovery of microvesicles (MVs). In 1967, while investigating the mechanisms of blood coagulation, Peter Wolf described what he called "platelet dust".[2] This platelet dust, isolated by high-speed centrifugation, showed procoagulant activity, and was thought to explain the mystery of platelet-free plasma coagulation. Wolf's "dust" was soon after termed microparticles, and later renamed microvesicles to avoid confusion with non-cellular microparticles.

In the fifty years since Wolf published his article describing platelet dust, a large body of research has expanded the understanding of these cellular packages. In 1970, Webber and Johnson took the first images of the formation and release of MVs from platelets.[3] They found that thrombin initiates the formation of what they described as concentric rings and hollow spheres within the platelet cytoplasm, which are released from the cell. Four years after Wolf's discovery, Crawford used electron microscopy to image platelet MVs (PMV), describing them as having a heterogeneous granular appearance, ranging between 20 to 40 nm in diameter.[4] Further, electron micrographs of platelets showed blebbing of the plasma membrane that was structurally similar to the granule-like PMVs. This observation, with the findings that the MV homogenates showed ATPase activity and contained actomyosin-like contractile proteins similar to those of platelets, led Crawford to hypothesize that PMVs originated from this membrane blebbing.

In 1987, Johnstone et al. described a new population of cellular vesicles while investigating erythrocyte production. They found that these small vesicles, termed exosomes, were released from reticulocytes (immature erythrocytes) and contained enzymes and transmembrane transporters characteristic of reticulocytes.[5] Since then, exosomes have been shown to be just as diverse in form and function as MVs.[6] In fact, platelets, among other cell types, have been shown to produce both MVs and exosomes.[7] These two populations are differentiated based on size and

mechanism of formation; MVs are defined as 100 nm to one µm plasma-membrane derived vesicles, while exosomes are less than 100 nm in diameter and are formed intracellularly (Figure 1).

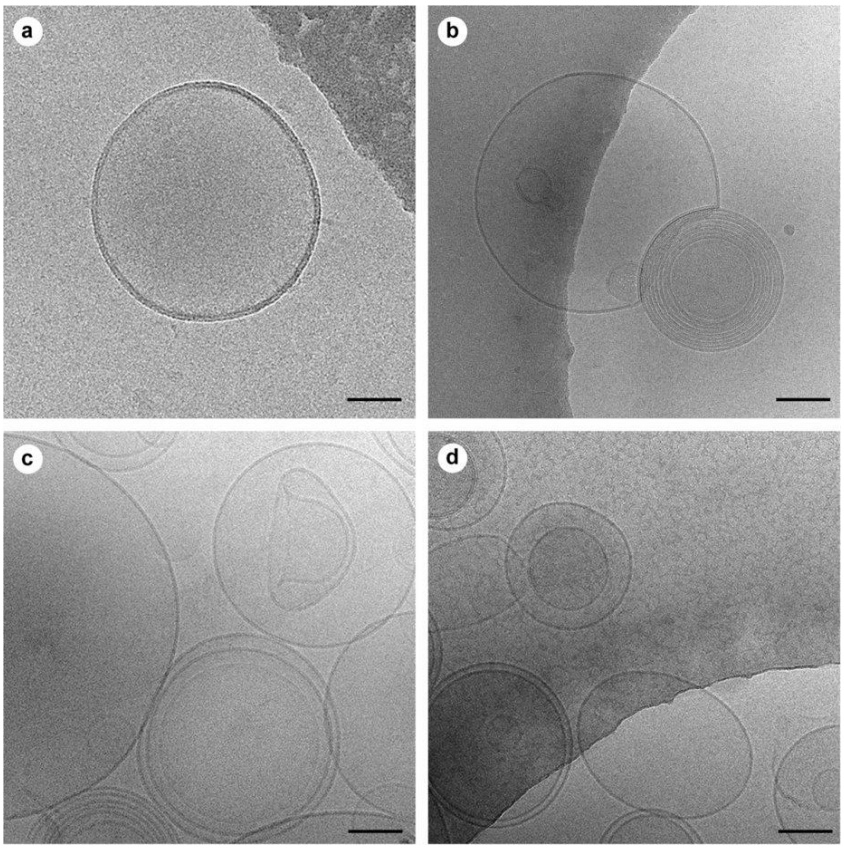

Figure 1. Cryo-electron micrographs of extracellular vesicles isolated by protein organic solvent precipitation with MVs (>100 nm) and exosomes (<100 nm) present. Scale bars = 55 nm. (Figure from Gallart-Palau et al. (2015)[8]).

An article published in 1977 suggested that membranous particles, isolated by ultracentrifugation or gel filtration of urine and cell culture media, contain both membrane-bound and cytoplasmic enzymes.[9] This finding illuminated the fact that these extracellular vesicles (EV) carry membrane and cytoplasmic contents of their parental cell. Indeed, EVs are bioactive components involved in intercellular communication and altering physiological processes and are often associated with disease states. MVs carry a variety of cytokines and signalling molecules that modify the phenotype and function of cells that they interact with through fusion, endocytosis or induction of signalling cascades. MVs also deliver mRNA and microRNA to target cells, where *de novo* translation or translational regulation induces changes in protein expression and cellular behavior.[10,11] MVs are derived from every cell type, including tumor cells, and are found in all

biological fluids, including breast milk,[12] cerebrospinal fluid,[13] semen,[14] urine,[15] and blood. The cargo carried by MVs is taken from the parental cell and transported throughout the body through the circulatory system, allowing stationary cell types to have far reaching effects. However, the cargo composition can be significantly different from the contents of the parental cell, depending on the agonist that induced MV formation, complicating the relationship between MV type and function.[11]

1.2 Microvesiculation

MVs are formed from many cells types, including leukocytes, erythrocytes, endothelial cells, and platelets (PMV), with PMVs making up the majority of the circulating MV population. MV formation is induced by cellular activation or apoptosis. In terms of PMVs, this activation may come in the form of collagen, Ca^{2+}, thrombin, norepinephrine, or shear stress.[16] In vitro production of PMVs is often conducted using thrombin, or thrombin receptor-activating protein (TRAP).[10,17] The processes behind the formation of MVs have been best characterized in terms of PMVs but the complete mechanism of how many agonists induce formation is unknown.

Activation begins with stimulus from an agonist, such as thrombin, binding to platelet surface receptors (Figure 2). The stimulus activates phospholipase C-β (PLC), which hydrolyses phosphatidylinositol 4,5-biphosphate (PIP_2) in the inner leaflet of the plasma membrane to yield membrane-bound diacylglycerol (DAG) and cytoplasmic inositol 1,4,5-triphosphate (IP_3). IP_3 triggers the release of Ca^{2+} from internal stores, in turn inducing the influx of extracellular Ca^{2+}.[18] This cascade results in a dramatic increase in cytosolic Ca^{2+} concentration, which causes cytoskeletal remodelling by a conformational change in cytosolic calpain, forming an active protease that cleaves cytoskeletal proteins.[19,20] Experiments that have artificially induced PMV formation through the application of a Ca^{2+} ionophore and TRAP, evoke Cdc42 and Rac1 G proteins, which mediate actin modification and cytoskeletal reorganization through the p21-activated kinase (PAK).[21] However, inhibiting upstream at calpain does not completely blunt PMV production, suggesting other mechanisms are involved.[21] Indeed, other agonists likely invoke different pathways. Regardless of the pathway involved, cytoskeletal degradation results in detachment of areas of the plasma membrane, which protrude from the cell and eventually bleb off to form MVs.

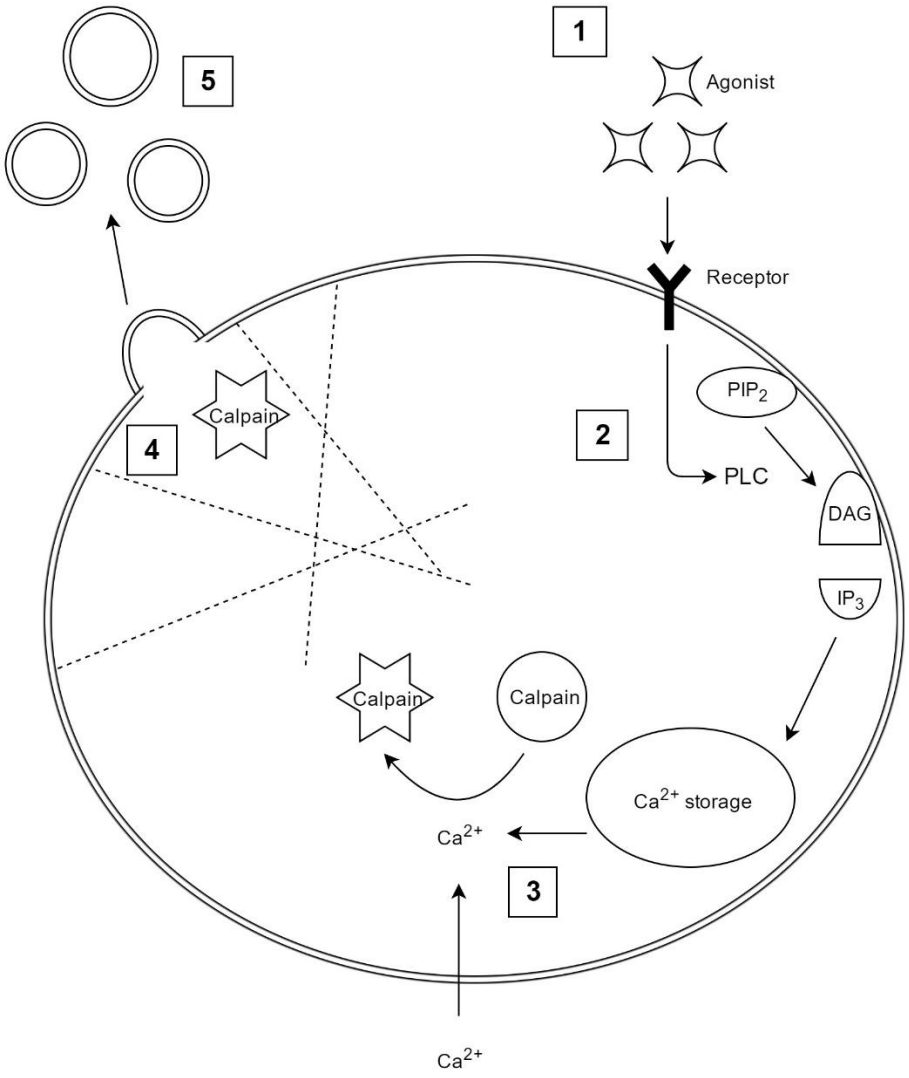

Figure 2. Pathway of platelet activation leading to microvesicle formation. Biogenesis begins by cellular activation (1), which initiates a PIP_2 signalling cascade (2) that results in an increase in the cytoplasmic Ca^{2+} concentration (3). Calpain is activated and degrades the actin cytoskeleton, disrupting the plasma membrane (4), inducing the formation of blebs that separate from the cell (5).

Phosphatidylserine (PS), an anionic phospholipid, is usually present on the inner leaflet of the plasma membrane; however, during platelet activation and MV formation, PS becomes externalized.[22] Ca^{2+} inhibits the transmembrane lipid transporter flippase, which is responsible for maintaining membrane asymmetry by inward PS translocation. The increase of cytosolic Ca^{2+} also activates scramblases, which allow for random movement of phospholipids between the inner and outer membrane leaflets.[22,23] Together, these two mechanisms result in PS expression on the outer membrane leaflet of MVs. While some studies have suggested that PS exposure is not characteristic of all MV populations, PS is assumed to be expressed on the surface of PMVs.[24]

1.3 Isolation and quantification of MV populations

As there are several methods used to isolate MVs rather than one standard procedure, reported MV concentrations and population characteristics are highly variable between studies. Centrifugation is a simple method of isolation, usually using two centrifugation steps to remove first the blood cells, and then platelets, with a third high-speed centrifugation to pellet MVs. While centrifugation is the most widely used isolation method throughout literature, there is no consistent centrifugation speed and time, leading to potentially inconsistent results between different labs. An attempt was made by the Society of Thrombosis and Haemostasis to standardize the process of blood collection and MV isolation.[25] This report highlights the importance of not just consistent isolation methods, but handling of samples, as factors such as the blood draw procedure, the choice of anticoagulant, freezing, and the time before processing can affect the MVs in the sample.[25] However, many studies published after the release of this report do not comply with the standardized procedure. Centrifugation can be paired with density gradients and filters to improve sample purity, but these methods may reduce the MV yield, making the results difficult to compare to similar studies.[26]

A novel method of protein organic solvent precipitation (PROSPR) enables effective purification of intact MVs and exosomes in plasma by removing soluble proteins with acetone.[8] While this method is ideal for analysis of cargo proteins, nucleic acids and lipids, it is not appropriate for functional assays because the acetone precipitation may denature functional surface markers used to identify MV populations. Another method that is useful for characterization, but not for functional studies, is the use of magnetic beads conjugated to MVs with specific binding proteins, such as annexin V, lactadherin or MV antigen specific antibodies.[27] Because MVs carry surface markers from their parental cell, they can be characterized in terms of their origin. PMVs typically carry CD41 (integrin α2β), leukocyte MVs can be identified by exposed CD45 (protein tyrosine phosphatase, receptor type C), and endothelial MVs carry both CD144 (vascular cell adhesion protein 1) and CD62E (E-selectin).[28] The bead-bound MVs can be easily isolated with a magnet. However, the addition of the magnetic beads is non-reversible and could potentially interfere with interactions between cells and MVs. This method further suffers from poor yield and nonspecific binding.

These methods have the disadvantage of not differentiating between MVs and exosomes. Even with size-gating, MV and exosome populations cannot be completely separated due to overlap in size. As such, it is nearly impossible to investigate the characteristics and functions of just MVs or exosomes, and rather EVs are better characterized by surface markers. Also, because MV and exosome populations overlap in size, methods that count MVs based on size may over estimate MV concentrations.

As with isolation, there is no standard methodology for MV enumeration and these methods do not reliably differentiate between MVs and exosomes, resulting in inconsistent estimates of MV concentration. Flow cytometry is the most commonly used method of enumerating MVs. However, this approach has a drawback in that small MVs and exosomes are not reliably detected by previous generation flow cytometers.[29] To detect the smaller MVs, they can be conjugated to beads that increase light scattering, but, as described above, this method prevents the MVs from being used in subsequent functional studies. However, new generation flow cytometers enable detection of MVs smaller than 200 nm, which has increased accurate MV quantification.[30] In fact, when using flow cytometers that can detect vesicles in this range, the calculated MV concentration increases dramatically.[31] Nanoparticle tracking analysis, in which a laser monitors the Brownian motion of small particles, is also used for MV quantification and can measure the size of individual MVs.[32] This method is effective at detecting MVs 70 nm and larger, and, with the incorporation of antigen-specific fluorescent labelling, MV subsets within a sample can be differentiated.[32]

1.4 Mechanisms of interaction

MVs interact with target cells through many pathways. MVs may induce signalling cascades by binding receptor proteins on the surface of target cells, or may fuse to, or be taken up by, the target cell.[33] MVs may also lyse, releasing cargo into the blood, allowing the contents to interact with cell surface receptors. Regarding PMV interactions with endothelial cells, the majority of research indicates MVs are taken up via receptor-mediated endocytosis. The mechanisms that induce endocytosis of MVs are unclear and many pathways have been proposed. Membrane lipids, specifically PS, and endothelial surface receptors have been implicated in several pathways.[34,35] Baj-Krzyworzeka et al. (2002) found that degradation of PMV surface proteins with trypsin or proteinase K increases MV uptake by endothelial cells, indicating that PS is mediating the interaction and removal of surface proteins allows for easier access of PS to receptors.[36] However,

Faille et al. (2012) found that trypsin degradation decreased PMV endocytosis by endothelial cells, suggesting that MV surface proteins are involved in uptake.[34] These results highlight an important aspect of MVs; the characteristics of MVs are dependent on the stimulus of production as well as the cell of origin. While Baj-Krzyworzeka et al. (2002) activated platelets with thrombin and collagen, Faille et al. (2012) used a calcium ionophore.

Developmental endothelial locus-1 (Del-1), a glycoprotein secreted by endothelial cells, has been shown to be involved in PMV clearance from the circulatory system by bridging MVs and endothelial cells.[37] Del-1 contains binding domains for both integrin αvβ3, which is expressed on the endothelial plasma membrane, and PS, thus allowing endothelial cells to bind PS-expressing MVs.[38,39] Antibody inhibition of Del-1, annexin V blockade of PS and pharmacological inhibition of clathrin with chlorpromazine all decrease the uptake of platelet MVs by endothelial cells, implicating Del-1 in clathrin-dependent endocytosis of PMVs.[37] Similarly, lactadherin, an opsonin secreted by endothelial cells into the blood, mediates PMV internalization by endothelial cells by binding MV phosphatidylserine and endothelial integrin αvβ3.[17] Interestingly, MVs from apoptotic endothelial cells have been shown to express annexin V, which mediates uptake by binding PS on the outer leaflet of apoptotic endothelial cells.[35]

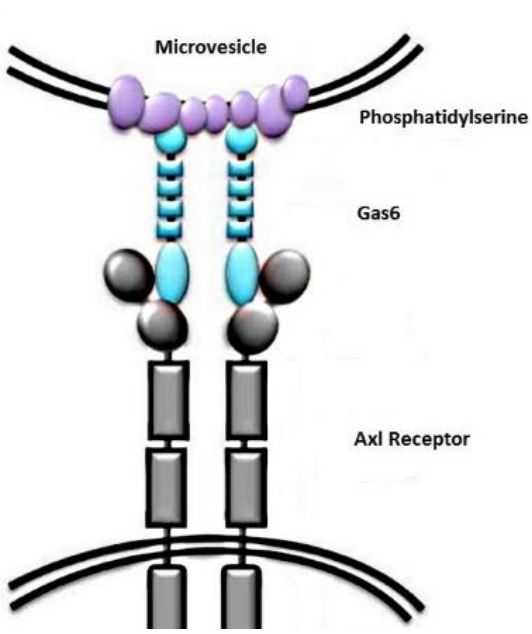

Figure 3. The mechanism of endothelial cell and MV binding via Axl/Gas6. Image from Federation of American Societies for Experimental Biology (http://www.fasebj.org/content/30/1_Supplement/1117.1/F1.expansion.html)

Of particular interest to the current study is the TAM (Tyro3, Axl and Mer) receptor, Axl, and its ligand, Gas6. A recent report indicated that Gas6 bridges PMVs and endothelial cells by binding both Axl, expressed on the surface of endothelial cells, and PS on PMVs (Figure 3).[40] Gas6 is produced by endothelial cells and is found in plasma at a concentration of 13 to 23 ng/mL.[41,42] TAM receptors are important for clearance of apoptotic cells by macrophages and dendritic cells, and because MVs share characteristics with apoptotic cell fragments, such as externalized PS, this pathway may be involved in the clearance of MVs by endothelial cells.[43] This Axl/Gas6 interaction

results in PMV internalization by human umbilical vein endothelial cells (HUVECs) and the addition of anti-Axl antibody inhibits this uptake.[40]

While multiple PMV internalization mechanisms by endothelial cells have been reported, the mechanism has not been investigated in models involving exercise-derived MVs. However, it is probable that mechanisms are not exclusive to exercise-derived MVs, as agonists typical of an exercise state, such as thrombin, collagen, and shear stress, are upregulated during coagulation cascades and other physiological responses.

1.5 Effects of microvesicles on endothelial cells

The effect of MVs on the endothelium, the lining of endothelial cells that covers blood vessel walls, has not been extensively investigated. However, there have been several papers that suggest deleterious effects from MV populations associated with disease states. MVs from apoptotic T cells and from patients with metabolic disease increase *in vitro* endothelial dysfunction by inducing endoplasmic reticulum stress and activating the unfolded protein response, which increases cytoplasmic reactive oxygen species and decreases nitric oxide synthase activity.[44] This response increases cellular stress and reduces the ability of endothelial cells to induce vasodilation.[45] Disease states, such as late-stage pulmonary arterial hypertension (PAH), increase circulating MV concentrations, and these MVs stimulate intracellular adhesion molecule-1 (ICAM-1) expression by cultured pulmonary arterial endothelial cells.[46] This may be an adaptive response, as ICAM-1 aids immune cells in infiltrating lung tissues, but the increase in inflammatory cytokines released by leukocytes can cause deleterious complications.[46] A high-fat diet leading to obesity in rats increases circulating concentrations of endothelial, platelet, monocyte and T-cell MVs.[47] These MVs upregulate the expression of vascular cell adhesion molecule-1 and reactive oxygen species in cultured endothelial cells, similar to that seen in late-stage PAH.[47] Further effects of monocyte-derived MVs include *in vitro* induction of endothelial cell apoptosis by delivering active caspase-1, a protease that has been shown to induce apoptosis through the NF-κB signalling pathway that is involved in transcriptional regulation of genes such as *TNFα* (tumor necrosis factor), *TNFβ* and *β-Interferon*, indicating that MVs may be involved in regulation endothelial cell survival.[48,49]

While MVs can have deleterious effects on the endothelium, they can also have adaptive effects. MVs can induce angiogenesis, which involves proliferation and migration of endothelial cells. CD40 ligand positive MVs isolated from atherosclerotic lesions induce neovascularization

of plaques by binding CD40 on endothelial cells *in vitro*, which results in expression of several angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor.[50] Although formation of the vasa vasorum in atherosclerotic plaques is detrimental and may cause lesion rupture, this mechanism may be active in other regions of the cardiovascular system where angiogenesis is beneficial, such as in muscle tissue. PMVs improve the ability of circulating angiogenic cells (CAC), isolated from atherosclerotic patients, to adhere to the endothelium, after which they migrate into the intercellular spaces between endothelial cells and release pro-angiogenic cytokines.[51] PMV-treated CACs also increase neovascularization when administered to rats with hind limb ischemia, improving oxygenation of ischemic tissues. This effect has also been seen with rats treated with PMVs from healthy donors, suggesting that PMVs may be interacting with CACs *in vivo*, or are inducing neovascularization of ischemic tissue through a separate mechanism.[51,52] PMVs may induce angiogenesis through activation of phosphoinositide 3-kinase by releasing VEGF that binds to surface receptors, initiating a signaling cascade that results in endothelial proliferation, migration and angiogenesis, both in *in vitro* and *in vivo* models.[52] However, endothelial MVs have the opposite effect, reducing the length of new capillaries and decreasing the number of branch points in capillary networks *in vitro*.[53] A recent study linked exercise-derived MVs, consisting primarily of PMVs, to increased proliferation, angiogenesis and wound healing of cultured endothelial cells and it is possible that this is due to an increase in platelet derived MVs.[54]

1.6 Microvesicles and exercise

High intensity exercise has been shown to increase circulating MV concentrations, but the findings are somewhat inconsistent between studies. Chaar et al. (2011) found that strenuous exercise increased total circulating MV concentrations from 1000 MVs/µL to 4000 MVs/ µL and this increase persisted for two hours after cessation of exercise.[55] Further, this increase was attributed mainly to PMVs, while erythrocyte, monocyte, and endothelial MV populations did not increase. Studies from Sossdorf et al. (2010) and Maruyama et al. (2012) have identified similar trends in MV dynamics, although the magnitude of exercise-derived PMV increase varied from a nearly three-fold increase to a moderate increase of less than 25%, respectively.[56,57] Another study focused on exercise-derived EVs between 100 and 300 nm, which includes exosomes as well as small MVs.[58] Similar to larger MVs, the concentration of these small EVs increased 2.7-fold,

although the increase was not maintained at 90 minutes post-exercise. On the other hand, Mobius-Winkler et al. (2009) identified no significant increase in total circulating MV concentrations or in the specific endothelial MV population after exercise.[59] The experimental design included exercise at 70% of anaerobic threshold, while studies that found significant increases in MV concentrations used 80 to 85% of maximal aerobic power or, in the case of Chaar et al. (2011), repeated maximal exercise designed to simulate training sessions. It is possible that high enough exercise intensity was not achieved in the Mobius-Winkler et al. (2009) study, but a minimal exercise intensity necessary to induce MV production has not been determined.

Although the mechanism behind the exercise-derived MV increase is not completely understood, explanations include a reduction in plasma volume, an increase in platelets and leukocytes as well as an increase in MV agonists.[56] Exercise causes plasma to migrate into the extravascular tissue and contracting muscle, reducing plasma volume and increasing the relative concentration of blood constituents,[60] including red blood cells, leukocytes and platelets.[61,62] As exercise intensity increases, blood concentrations of many known platelet agonists, such as norepinephrine and ADP, increase substantially. As well, increased heart rate and blood pressure increase shear stress and these combined factors may be responsible for the increase in circulating MVs. This is further supported by the increase in PS-expressing MVs, which are generally assumed to be of platelet origin.[56]

1.7 Therapeutic potential

While much research has indicated harmful effects of MVs, a new focus has been on using MVs for the treatment of various diseases, such as cancer. MVs are ideal for therapeutic applications; MV membranes are stable and resistant to lysis, *in vitro* preparation is relatively simple, and they benefit from being endogenous and thus have a low risk of inciting an immune response. A recent article showed that tumor-derived MVs, when taken up by bladder tumor cells, increase sensitivity to chemotherapy by reducing efflux of the drugs and inducing entry of the drugs into the nucleus.[63] While this treatment prevented tumor growth in rats and increased survival rate, it carries risk, as tumor-derived MVs are also involved in the modulation of the tumor microenvironment and supporting tumor growth.[64]

MVs have also been investigated for application as a drug delivery system. Through electroporation, MVs were loaded with siRNAs and chemotherapy medication and were injected

into mice with breast cancer, effectively reducing tumor size.[65] This study highlights the versatile applications of MVs for drug delivery, as MVs were engineered with surface markers to target them to specific cells. Although these studies are promising, MVs are still poorly understood and further characterization of cargo and pathophysiology are required before therapeutic applications are possible in humans.

2 INTRODUCTION

Exercise is important for maintaining proper health and preventing cardiovascular disease (CVD) by significantly reducing associated risk factors. High dose lipid lowering drugs and blood pressure medications are effective at reducing risk factors for CVD, but do not prevent CVD as effectively as regular exercise.[66] However, reduction of CVD risk factors, such as obesity, hypertension and diabetes, only accounts for 60% of the protective effect of exercise, leaving 40% unexplained (reviewed in Joyner and Green[66]). An important exercise-derived adaptation is improved endothelial function.[67] Endothelial cells form a confluent monolayer covering the inner surface of blood vessels and act as the first point of contact between blood and tissues. The endothelium is important for coagulation, inflammation, vasodilation and angiogenesis.[68] As with generalized exercise-derived adaptations, the mechanisms behind the improvement of endothelial function are not fully understood.[67] Recently, improved endothelial function due to exercise has been linked, in part, to microvesicles (MV) released during exercise.[54]

MVs are extracellular vesicles that range from 100 nm to one μm in diameter and are released from the plasma membrane in response to activation or apoptosis. They are present in all biological fluids, but are best characterized in blood. There are several populations of MVs in the circulatory system, derived from erythrocytes, leukocytes, endothelial cells, and platelets, with platelet MVs accounting for the majority.[69] Within the circulatory system, MVs have many known functions, such as participating in hemostasis, angiogenesis, and cell-to-cell communication.[70,71] They carry surface markers and cytokines derived from their cell of origin and modulate the biological function of cells they interact with.[72] MVs also carry mRNA and microRNA, indicating the potential of impacting gene expression in target cells.[10] While MVs have many important functions, they are also associated with several disease states, such as cancer, inflammation and atherosclerosis.[73–75] The function of MVs varies depending on the cell of origin and stimulus of formation, resulting in a highly variable and versatile method of cellular communication.

Several studies have found that exercise increases the concentration of circulating MVs, especially platelet MVs, by about two fold.[55–58,76] Exercise causes several immediate physiological changes, including increased blood pressure, an increase in adenosine diphosphate (ADP), and release of cytokines such as IL-6, all of which have been shown to induce vesiculation.[77,78] A recent study suggests that exercise-derived MVs improve endothelial function, but subsequent

studies have not been conducted.[54] However, it has been proposed that exercise-derived MVs may be partially responsible for the protective effects of exercise, especially when considering endothelial cell health. How exercise-derived MVs differ from other MV populations is unknown and warrants further investigation.

MVs may interact with target cells through external induction of signal transduction pathways by MV surface proteins or proteins released from MVs. However, endothelial cells have been shown to commonly take up MVs through receptor-mediated endocytosis.[17,34,79,80] Leukocytes have also been shown to take up MVs, and accordingly MV concentrations quickly return to baseline due to this clearance after exercise.[81,82] How MVs interact with target cells is as variable as their function. Generally, MV formation is accompanied by PS exposure on the outer leaflet of the plasma membrane. Exposed PS is involved in many uptake pathways of MVs, typically involving a receptor/ligand pair, the ligand of which binds PS, or direct interaction between PS and the target cell receptor.[35,37,39,80] Most recently, the TAM receptor Axl, and its ligand Gas6, which binds PS, have been implicated in MV uptake.[40] TAM receptors are best characterized in the phagocytosis of apoptotic cell particles by macrophages, and, because endothelial cells both secrete Gas6 and express surface Axl receptor, it is thought that this pathway could facilitate MV uptake by the endothelium.[42] MVs also carry similar surface proteins to their cell of origin. In the case of platelet MVs, these surface proteins mainly consist of integrins and other adhesion proteins.[83] These integrins allow platelets to adhere to proteins of the extracellular matrix, such as collagen.[84] Platelet MVs may also be able to interact with endothelial cells through integrin proteins, although whether this mechanism leads to uptake is unknown. The mechanism by which exercise-derived MVs interact with target cells has not been investigated previously and is thus an important question to explore.

3 OBJECTIVES

The purpose of this study was to investigate the role of exercise-derived MVs in the protective effect of exercise on the endothelium. There were two overarching objectives for this project: 1) to determine the effect of exercise-derived MVs on endothelial cell function; and 2) to explore the mechanism of interaction between exercise-derived MVs and endothelial cells. Two functional assays were used to assess how MVs affected endothelial cells in culture, focusing on endothelial proliferation and wound healing. Blockade studies were set up in tandem with the functional assays

to study potential mechanisms of interaction. To investigate a general mechanism, MV surface proteins were degraded with trypsin. To investigate a more specific mechanism, the interaction between Axl and Gas6 was inhibited with an anti-Axl antibody.

4 MATERIALS AND METHODS

4.1 Ethics approval

Ethical approval was obtained from the Thompson Rivers University Research Ethics Board before human exercise trials began (Appendix 9.1). All participants signed informed consent forms before taking part in the study and were financially compensated.

4.2 Exercise trials

Eleven healthy people between the ages of 18 and 39 participated in the exercise trial. Before the exercise trial, participants performed an incremental cycling test in which they exercised until exhaustion to determine their maximum output. After a five-minute warm-up with no added resistance, weight was added to the exercise bike in 0.2 kg increments to gradually increase the exercise intensity while the participants maintained 60 rpm. Throughout the test, heart rate was monitored by electrocardiography analyzed using Lab Chart software. Blood pressure and rate of perceived exertion (RPE) were taken at two minute intervals. The maximum power output was calculated as the product of the cycling speed (60 rpm) and the weight added to the exercise bike at the point of exhaustion.

During the second visit, participants exercised on the exercise bike for one hour at approximately 60% of the max power output achieved during the first visit. A couple of participants were unable to maintain 60 rpm at 60% of the max power output and the intensity was adjusted accordingly. Heart rate was monitored throughout exercise and blood pressure and rate of perceived exertion were taken at 15 minute intervals. Blood lactate levels were measured with a Lactate Pro portable blood lactate analyzer every 15 minutes during exercise to ensure participants were exercising at a high intensity. After an hour of exercise, participants rested for an hour, while blood pressure and heart rate continued to be monitored. Lactate was measured again after an hour of recovery.

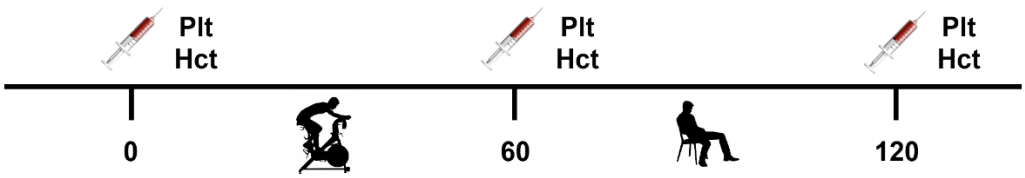

Figure 4. Exercise trial set up. Participants exercised for 60 minutes and then rested for 60 minutes. Blood was drawn and platelet count and hematocrit were measured at 0, 60 and 120 minutes. Plt = platelet; Hct = hematocrit.

4.3 Blood processing and microvesicle isolation

Blood was processed and MVs were isolated in accordance with the standardized protocol produced by the Society on Thrombosis and Haemostasis.[25] Using an 18-gauge needle, 30 ml of venous blood were drawn by Dr. Mark Rakobowchuk, a certified phlebotomist, at three times points during the second visit: before (rest), after (exercise) and an hour after (recovery) exercise (Figure 4). Platelet count and hematocrit were determined within one hour of drawing blood. Four and a half ml aliquots of whole blood were transferred to tubes containing 0.5 ml of 0.109 M sodium citrate anticoagulant. The blood was centrifuged at 2500 x g for 15 minutes at room temperature to remove blood cells. The platelet poor plasma was transferred to 1.5 ml Eppendorf tubes and centrifuged again at 2500 x g for 15 minutes at room temperature to remove platelets. The MV-containing supernatant was transferred to new Eppendorf tubes and was flash frozen in liquid nitrogen and then stored at -80°C.

4.4 Culture of human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVECs) pooled from multiple donors were used for the proliferation assay (Promocell C-12208). Immortalized HUVECS (Ea.hy 926 cell line, ATCC CRL-2922) were used for the scratch wound healing assay. HUVECs were cultured in M199 medium supplemented with 20% fetal bovine serum, 1% penicillin/streptomycin (AMRESCO K952-100ML), 0.5% 3 g/L endothelial cell growth supplement (Alfa Aesar BT-203), 2% 1 M HEPES buffer, and 0.2% 2 mM pyruvate. Cells were grown in T75 flasks and incubated at 37°C with 5% CO_2. Cells between passage three and five were grown to near-confluence and serum starved before use in assays. After removal from flasks with trypsin/EDTA, cell counts were performed using a hemocytometer and HUVECs were diluted to the proper concentrations for both assays.

4.5 Microvesicle treatment

MV samples were thawed on the day of experiments or the night before. They were centrifuged at 17,000 x g for one hour to pellet MVs and the plasma supernatant was discarded, leaving 60 µL above the pellet. MVs were washed with PBS and centrifuged again at 17,000 x g for one hour. The supernatant was transferred to a clean Eppendorf tube and used for control treatments. For MV surface protein blockade, 60 µL MV samples from each time point were treated with 100 µL 0.25% trypsin/EDTA. The MVs were incubated for 30 minutes at 37°C before trypsin deactivation with growth medium containing 20% serum. The trypsin-treated MVs (tMV) were pelleted by centrifugation at 17,000 x g, the supernatant was discarded, leaving 60 µL above the pellet, and the pellet was resuspended. MVs were diluted to physiological concentrations for both assays.

4.6 Proliferation assay

All treatments were tested in triplicate with samples from six participants. The proliferation assay was conducted as described in Wilhelm et al. (2016).[54] Ninety-six well plates were coated with 0.1% gelatin. Near-confluent HUVECs were remove from T75 culture flasks and resuspended at 40,000 cells/mL in serum free media. Fifty µL of cell suspension were added to each well along with another 50 µL serum free media and the appropriate MV treatment. For blockade of Axl/Gas6, the cell suspension was pre-incubated with anti-Axl antibody (R&D Systems AF154) or control IgG antibody (R&D Systems AB-108-C) for 30 minutes before addition to wells. The plates were incubated for four days at 37°C.

The medium was removed and wells were washed with 100 µL PBS before addition of 100 µL phenol-red free medium. Ten µL of 12 mM MTT reagent (thiazolyl blue tetrazolium bromide) (Sigma-Aldrich M5655) were added to each well and plates were incubated for three hours at 37°C. After incubation, 80 µL of medium were removed from each well and 50 µL of dimethyl sulfoxide were added and plates were incubated for 10 minutes at 37°C. The absorbance of each well was measured at 540 nm and 620 nm using a Multiskan Ascent® plate reader (Thermo Electron 1507540).

4.7 Scratch wound-healing assay

Treatments were tested in duplicate with samples from six participants, except for the tMV treatment which used samples from only five participants. Forty-eight well plates were coated with 0.1% gelatin. Cells were removed from T75 culture flasks and resuspended in growth medium to 50,000 cells/mL. Two hundred and fifty µL of cell suspension were added to each well and cells were cultured for six days until reaching confluence.

HUVECs were serum starved over night before the day of the assay to ensure that cells would be in the same stage of the cell cycle at the beginning of the assay. Using a 200 µL pipet tip, a straight line was scratched down the middle of each well, removing part of the HUVEC monolayer. For Axl/Gas6 blockade, wells were treated with 1.25 ug anti-Axl antibody and incubated for 30 minutes at 37°C before wells were scratched. Wells were washed with PBS and imaged at baseline with a microscope camera using ToupView software before addition of MVs or supernatant. Wells were imaged again after two hours of incubation. Images were analyzed with ImageJ software to determine the area of the scratch wounds in pixels.

4.8 Data analysis and statistics

Values are reported as mean ± standard deviation. Missing data points from exercise trials were extrapolated or interpolated from mean values assuming a linear relationship between time points. Because the tMV data were missing from one participant in the scratch wound healing assay, a sample was added using the calculated mean from the five participants to achieve a sample size of six.

Statistical analyses were performed with SPSS software. Exercise data were analyzed by one-way repeated measures ANOVA (time) and assay data were analyzed using two-way repeated measures ANOVA with condition (MV, supernatant, anti-axl antibody, tMV) and time (rest, exercise, recovery) as factors. If a significant main effect or interaction was noted, a Sidak post hoc test was subsequently performed to identify specific differences between means. Significance for all analyses was set at $P \leq 0.05$.

5 RESULTS

5.1 Maximum power output

This study involved eleven participants with an average age and body mass index (BMI) of 24.8 ± 6.4 years and 23.9 ± 3.1 kg/m^2, respectively. To determine maximum power output, participants exercised on an exercise bike until exhaustion, reaching an average maximum work rate of 245.3 ± 67.9 W. Participants achieved an average maximum heart rate of 189 ±8 beats per minute and an average maximum RPE of 8.6 ± 0.9 during the first visit (Table 1).

Table 1. Participant characteristics from the initial visit used to determine optimal output for second visit.

Participant	Age	BMI	Max Output (W)	Max Heart Rate (beats per minute)	Max RPE /10
01	20	25.53	120	-	9
02	18	20.38	168	191	6.5
04	25	25.38	228	195	10
05	37	20.50	322	173	9
06	20	24.37	252	183	8
07	26	24.97	240	198	9
08	20	30.85	300	195	9
09	22	23.00	300	195	8
10	23	25.76	288	183	9
11	36	22.54	312	185	9
12	26	20.10	168	190	8.5
Average ± SD	24.8 ± 6.4	23.94 ± 3.1	245.3 ± 67.9	189 ± 8	8.6 ± 0.9

5.2 Exercise trials

As it was important to ensure that participants exercised at an adequate intensity to stimulate MV production during the exercise trial, heart rate, blood lactate levels, hematocrit and RPE were monitored throughout the hour of exercise (Table 2). Heart rate peaked at an average of 170 ± 10 bpm (Figure 5D). Heart rate increased significantly within 15 minutes and remained elevated throughout exercise ($P < 0.01$), before returning to baseline 15 minutes after exercise.

Blood hematocrit increased by 2.6% with exercise but this increase was not found to be statistically significant after exercise (P = 0.06) or after recovery (P = 0.11) (Figure 5A). Lactate levels also increased with exercise (P < 0.01), peaking at 8.2 mmol/L after 30 minutes of exercise (Figure 5C). Some participants were not able to maintain 60% of max output for the whole hour and the intensity decreased during the last 15 minutes of exercise. This is reflected in a slight decrease in lactate at 45 minutes, but lactate levels remained significantly increased compared to baseline. Mean platelet levels rose by 36.6% during exercise and were maintained throughout the hour of exercise and into recovery (P = 0.02) (Figure 5B). RPE reflected moderate activity after 15 minutes of exercise and increased to reflect vigorous activity by 45 minutes. The mean maximum RPE reported during the exercise trial was 0.6 points less than the mean maximum RPE reported during the first visit (Figure 5E).

Table 2. Variables monitored throughout exercise trials. Values are mean ± SD. (-) indicates times at which variables were not measured.

	Baseline	Exercise				Recovery			
Time (minutes)	0	15	30	45	60	75	90	105	120
Hematocrit (%)	46.3 ±3.2	-	-	-	48.9 ±3.8	-	-	-	48.9 ±1.7
Platelet (10^4 per µL)	5.7 ± 2.6	-	-	-	8.6 ±2.3	-	-	-	7.1 ±1.7
Lactate (mmol/L)	1.7 ±0.5	7.3 ±3.0	8.2 ±2.4	7.7 ±2.5	4.2 ±1.6	-	-	-	1.8 ±0.4
Heart rate (BPM)	84 ± 16	159 ±15	168 ±12	167 ±11	169 ±8	93 ±15	79 ±8	77 ±8	80 ±12
RPE	0	5.4 ±1.1	6.9 ±0.7	7.5 ±1.3	8.0 ±0.8	-	-	-	-

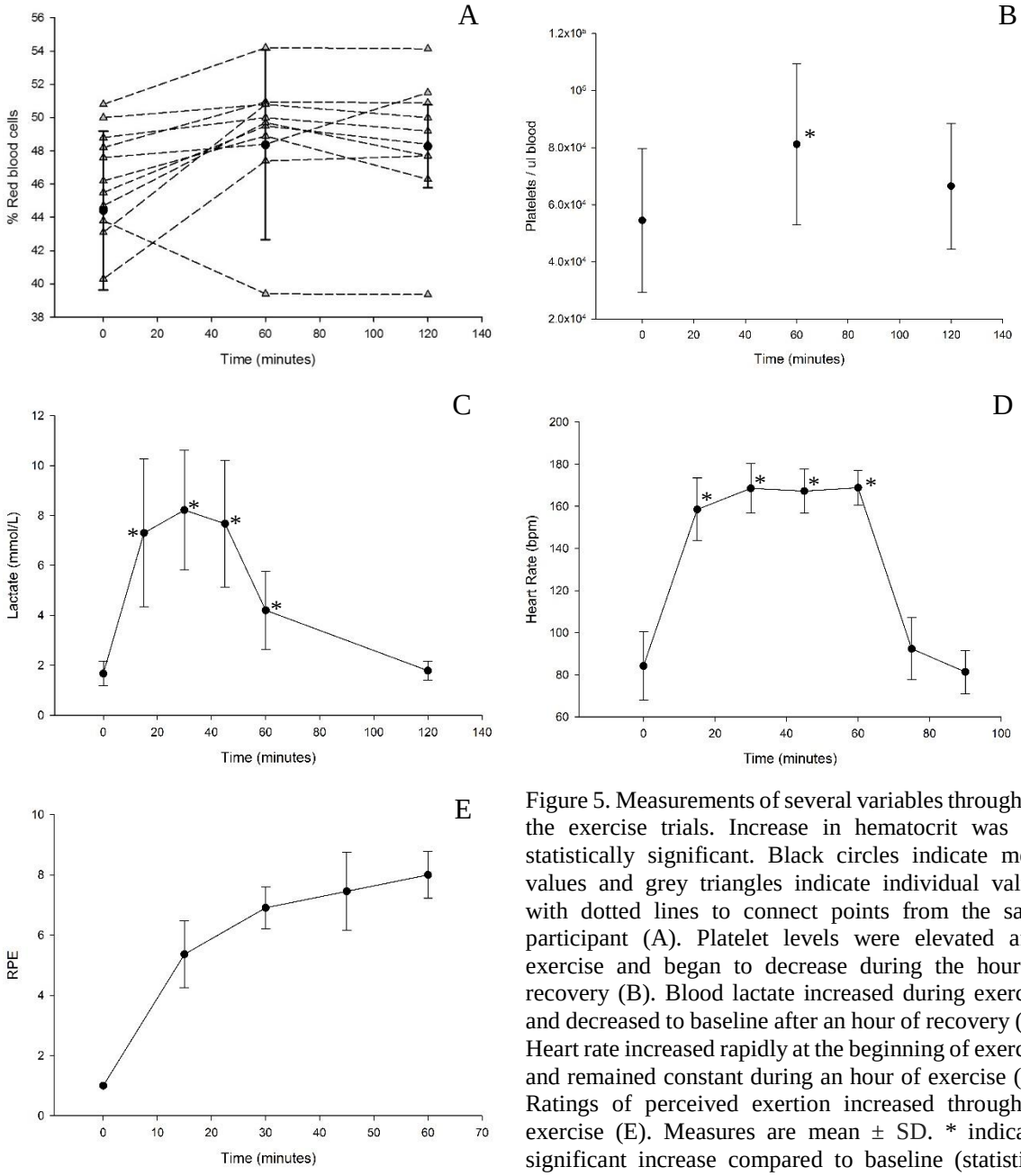

Figure 5. Measurements of several variables throughout the exercise trials. Increase in hematocrit was not statistically significant. Black circles indicate mean values and grey triangles indicate individual values with dotted lines to connect points from the same participant (A). Platelet levels were elevated after exercise and began to decrease during the hour of recovery (B). Blood lactate increased during exercise and decreased to baseline after an hour of recovery (C). Heart rate increased rapidly at the beginning of exercise and remained constant during an hour of exercise (D). Ratings of perceived exertion increased throughout exercise (E). Measures are mean ± SD. * indicates significant increase compared to baseline (statistical analysis was not run for RPE data).

5.3 Proliferation assay

To determine the effect of exercise-derived MVs on the proliferation of endothelial cells, HUVECs were treated with MVs or supernatant from rest, exercise and recovery. The cells were then cultured in 96-well plates for four days before an MTT assay was used to determine the relative concentration of cells (Figure 6). Because the yellow MTT reagent is reduced by mitochondrial reductases to produce purple formazan, an increase in absorbance correlates to an increase in cell density. No significant differences were observed between the absorbance measured for MV treatment and supernatant controls ($P = 0.62$). Exercise MVs did not have a significant effect on proliferation ($P = 0.12$).

Degradation of MV surface proteins and inhibition of the Axl/Gas6 interaction were used to investigate the mechanism of interaction between HUVECs and exercise MVs. The tMV treatment showed significantly more HUVEC proliferation than the MV treatment ($P = 0.02$) and supernatant treatment ($P = 0.02$) at all time points. Blockade with anti-Axl antibody resulted in increased proliferation of HUVECs over supernatant treatment ($P < 0.01$) but not over MV treatment ($P = 0.15$).

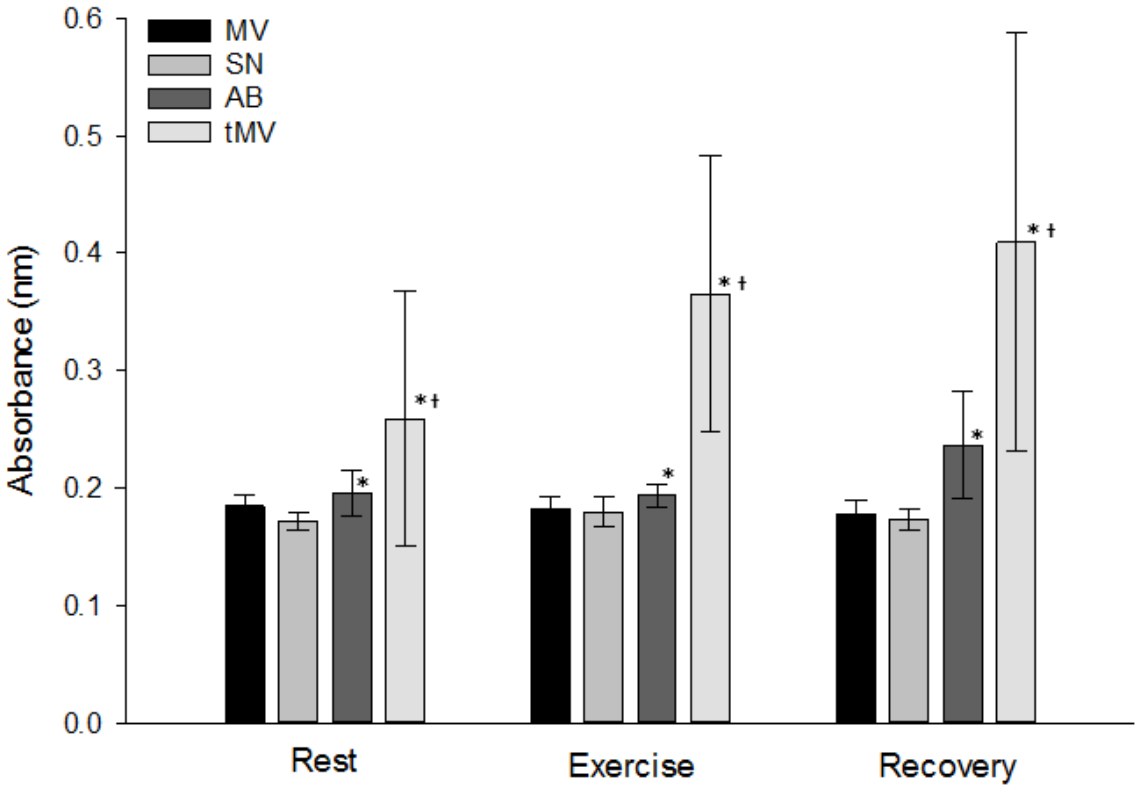

Figure 6. Average absorbance of different treatments measured at 540 nm for the proliferation assay. Averages were calculated from six samples run in triplicate. Measures are mean ± SD. MV = microvesicle; SN = supernatant; AB = anti-Axl antibody; tMV = trypsin-treated MVs. ǂ shows significant difference from MV treatment; * shows significant difference from supernatant treatment within time point.

5.4 Scratch wound healing assay

To determine the effect of MVs from different time points on the migration and wound-healing abilities of endothelial cells, a straight line was scratched in confluent monolayers of HUVECs. HUVECs were then treated with MVs or supernatant and wound closure was monitored over two hours (Figure 7). As with the proliferation assay, blockades were also set up to investigate the mechanism of interaction between endothelial cells and MVs. No significant differences in wound closure were observed between treatments with rest, exercise and recovery MVs ($P = 0.48$). However, there was a significant condition by time interaction where the resting MV treatment resulted in decreased migration of cells into the scratch wound than the resting tMVs ($P = 0.04$). Exercise MVs induced more wound closure when HUVECs were pretreated with anti-Axl antibody, compared to cells not exposed to the blockade ($P < 0.01$). Unexpectedly, treatment with supernatant from the recovery time point resulted in improved wound closure over recovery MVs

(P = 0.002). Treating HUVECs with tMVs from rest resulted in more wound closure than tMVs from recovery (P = 0.02).

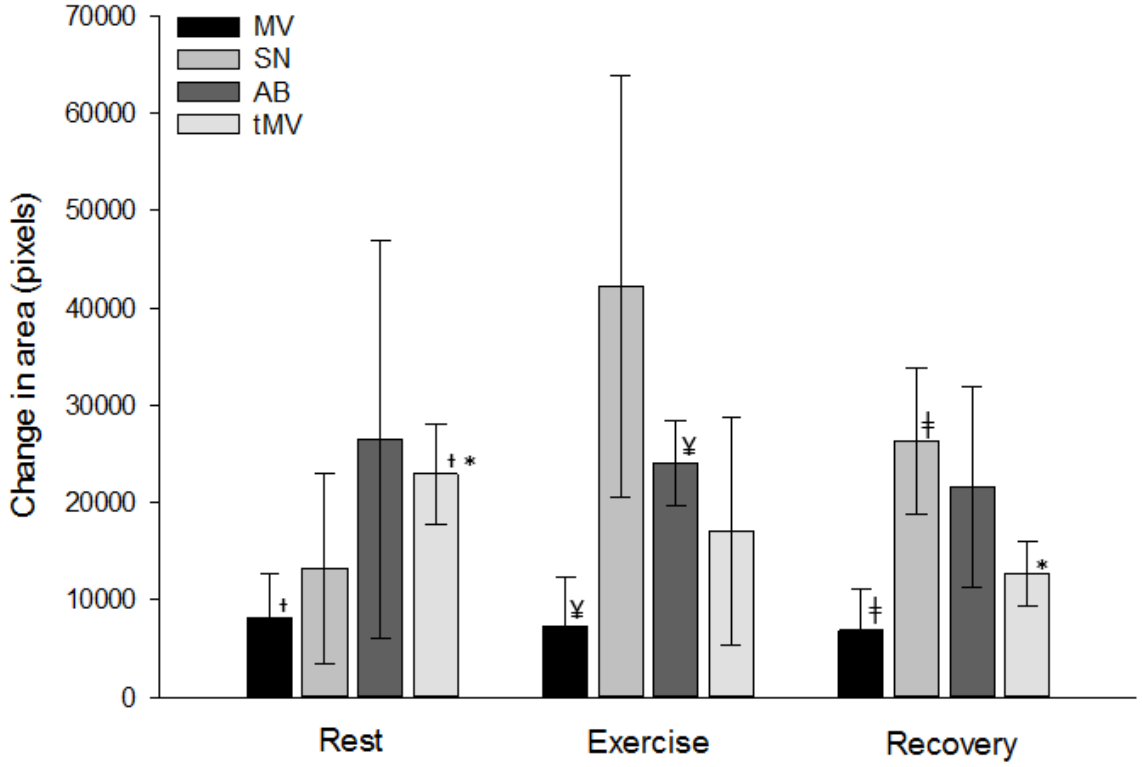

Figure 7. The change in area of HUVEC scratch wounds over two hours. Measures are mean ± SD. MV = microvesicle; SN = supernatant; AB = anti-Axl antibody; tMV = trypsin-treated MVs. Matching symbols indicate significant differences between treatments.

6 DISCUSSION

6.1 Exercise intensity

The purpose of the exercise trials was to have participants exercise at a high intensity to induce the production of MVs. Therefore, maximum work rates were determined during the participants' initial visit. During the exercise trial, participants exercised at 60% of their maximum work rate to ensure a high level of physical exertion that would be sustainable for the full hour of exercise. Several parameters indicative of exercise intensity were monitored throughout exercise to provide support for the attainment of a vigorous exercise stimulus previously highlighted as an important variable leading to MV appearance with exercise.

Heart rate may be used as an appropriate measure of exercise intensity, with values above 80% of maximal heart rate indicative of vigorous activity. The maximum average heart rate reached 170 ± 10 bpm during the hour of exercise, compared to the average maximum heart rate of 189 ± 8 bpm during maximum output testing, indicating that participants did not reach maximum output during the hour of exercise. However, 170 bpm does indicate intense physical exertion at ~90% of maximal, which would be considered within the heavy exercise domain and likely to induce MV formation.[54]

During high intensity exercise, the metabolite lactate is released from skeletal muscle cells. The average lactate level of 1.6 ± 0.5 mmol/L recorded at baseline is typical for individuals at rest.[85] The maximum average lactate level of 8.2 ± 2.4 mmol/L indicates that a steady state of high intensity exercise was achieved throughout the last 30 minutes, again suggesting participants exercised at a high enough intensity to produce MVs.[85] Lactate levels peaked at 30 minutes and then declined slightly during the second half of the exercise trial because some participants were exercising at an intensity too high to maintain for the entire hour. Although it was necessary to decrease the intensity of exercise for some participants, based on blood lactate data it can be concluded that participants were exercising at a high intensity throughout the hour.

During exercise, the red blood cell concentration increases due to fluid loss and lowering of plasma levels as plasma shifts to the extracellular compartment. This is an important adaptation to exercise, as red blood cells contribute to the buffering of blood pH by binding protons and CO_2, and supply tissues with oxygen.[86] Thus, measuring the percentage of blood composed of erythrocytes gives insight into the intensity of exercise. The observed increase in hematocrit during

the hour of exercise and after recovery was not significant, indicating that the participants were not exercising at a high enough intensity to induce fluid loss.

The reported RPE indicated that participants felt a relatively high strain during the hour of exercise. However, RPE is subjective and thus is not a measure of true exertion. Nevertheless, RPE does reveal information about the psychological state of participants, allowing researchers to adjust exercise parameters so that participants were able to complete a full hour of exercise.

Since exercise increases platelet concentrations and activates platelets through shear stress, it is expected that higher levels of platelets associated with exercise will result in an increase in MVs.[87] Platelet concentrations increased by 36.6% after the hour of exercise, suggesting that MV concentrations would have also increased. These changes in blood platelet concentrations mirrored those of Wang et al., who found that intense exercise increased platelet levels by 24.7%.[88]

6.2 Effect of exercise MVs on endothelial cell function

To investigate whether exercise-derived MVs improved endothelial function, two assays were used to assess the proliferative and wound closing abilities of HUVECs treated with MVs or supernatant collected from participants during rest, exercise and recovery. The results of this study are inconclusive when considering how exercise MVs affect endothelial function. There were no observed differences in HUVEC proliferation between treatments with MVs from rest, exercise and recovery, suggesting that either exercise does not increase MV concentrations or that the MVs produced by exercise have no inherent effect on the proliferative ability of endothelial cells. Similarly, MVs from exercise did not improve wound closure compared to MVs from rest or recovery. These results are in contention with a recent study that showed increased proliferation, migration and wound healing of endothelial cells treated with exercise MVs, as well as several studies linking PMVs to improved endothelial function and neovascularization.[50–52,54] Although it appears that the participants achieved a high intensity of exercise, it is possible that the exercise was insufficient for MV production. However, moderate exercise has previously been shown to induce the release of platelet MVs.[56]

Unexpectedly, at the recovery time point, the supernatant control improved wound closure over the MV treatment. The control treatment was the resulting supernatant after washing MV samples with PBS and should have contained relatively little protein or other constituents found

in plasma. As such, it was hypothesized that the supernatant would have little effect on endothelial cells. It is possible that the MV treatment had a negative effect on the endothelial cells due to the presence of pro-coagulant PMVs. During blood draw, an 18-gauge needle was used to avoid shear stress-induced platelet activation, but it appears that the sodium citrate anticoagulant did not work as unwashed MV samples and the plasma supernatant induced coagulation when added to the functional assays. This finding was not consistent between assays, however, with no difference in endothelial proliferation between MV and supernatant treatments, indicating that MVs negatively affect wound closure, but not proliferation. Although proliferation is necessary for wound closure, other processes, such as migration, are involved and the MV treatment may have been detrimental to these processes, resulting in reduced wound closure.

6.3 Mechanism of interaction between MVs and endothelial cells

To investigate the mechanism of interaction between endothelial cells and exercise-derived MVs, potential uptake mechanisms were inhibited. Previous studies have suggested that MV surface proteins, such as glycoproteins and integrins, may be involved in the receptor-mediated endocytosis of MVs by endothelial cells and it was hypothesized that these proteins might mediate the interaction between exercise-derived MVs and endothelial cells.[36] To inhibit interactions involving MV surface proteins, these proteins were degraded with trypsin. It was expected that if MV surface proteins mediate the interaction, degradation of these proteins would inhibit the interaction and dampen the effect of exercise-derived MVs on endothelial function. However, tMVs had the opposite effect, increasing proliferation regardless of whether the treated MVs were from rest, exercise or recovery. This effect was also seen in the scratch wound healing assay, as tMVs from rest increased wound closure over rest MVs. Previous studies have indicated a role of MV glycoproteins in MV interaction with endothelial cells, and so degradation of these proteins should reduce the effect of MVs.[34] However, MV phospholipids, specifically PS, have been implicated in mediating MV uptake and thus the degradation of surface proteins may make these phospholipids more accessible.[17] As trypsin degradation may destabilize MV membranes, it is possible that MVs lysed, rather than remaining intact. This would result in MV contents being released into the medium, rather than MVs being taken up by endothelial cells. Platelet MVs contain high concentrations of platelet-derived endothelial cell growth factor (PDEGF) and VEGF, which induces endothelial proliferation and migration, as well as a host of proteins involved in

signal transduction and cell communication.[83] Although the contents of exercise-derived MVs have not yet been characterized, it is possible that these proteins were present, and their release into the medium has a greater effect than MV uptake. This is probable when considering the effect of PDEGF and VEGF, as these growth factors bind to extracellular membrane-bound receptors, which induce a signal cascade that results in endothelial proliferation and migration. As such, internalization of MVs would fail to activate these pathways, especially if MVs are taken into lysosomes for disposal.

Such dramatic effects of tMVs on proliferation may have been due to microbial contamination of the assay. Although penicillin and streptomycin were used to control potential bacterial contamination, no antifungal agents were used. This lack of antifungal agent, combined with several instances of contamination throughout the project, indicates that the increased proliferation may actually be caused by microbes growing in the wells rather than HUVECs. It is unlikely that the trypsin was the source of contamination, however, as the stock was used throughout the project for removal of cells from flasks. Unfortunately, the plates were not examined by microscopy after incubation with the MTT reagent, nor were contaminants isolated, so fungal contamination was not confirmed.

A recent study has implicated Axl and Gas6 in the receptor-mediated endocytosis of PMVs by endothelial cells and it was thought that this pathway may be involved in the uptake of exercise-derived MVs.[40] An antibody specific to the endothelial surface receptor, Axl, was used to block the interaction between MVs and endothelial cells through Axl and Gas6 bound to MV PS. It was expected that, if the Axl/Gas6 pathway is active in the interaction between exercise-derived MVs and endothelial cells, blocking the Axl/Gas6 interaction would decrease any beneficial effects seen with exercise-derived MVs. As with the trypsin treatment, antibody treatment increased endothelial cell function under several conditions. Axl blockade resulted in increased proliferation over supernatant treatments, although no difference was found between antibody and MV treatments. Antibody treatment of HUVECs also increased wound closure when cells were treated with exercise MVs. It was hypothesized that blockade of the Axl/Gas6 interaction would decrease the effect of MVs on endothelial cells. However, inhibition of the Axl/Gas6 pathway may have prevented uptake and caused MVs to remain in the media where lysis could occur, producing results similar to that of the tMVs. It can be assumed that the antibody used in this experiment

inhibits the interaction between Gas6 and Axl as this antibody was used in a previous study that showed effective blockade.[40] It is possible that the Axl/Gas6 pathway is not involved in the uptake of exercise MVs. Connor et al. (2010) showed that only a small population (~10%) of circulating MVs in unstimulated platelet-poor plasma bind annexin V, which indicates PS exposure.[24] Further, when stimulated by ADP or TRAP, which are both agonists relevant in an exercise model, less than 25% of PMVs bind annexin V.[24] However, the percentage of annexin V binding varies greatly depending on buffer as well as stimulus. Nevertheless, this finding calls into question how prevalent PS+ MVs are and what mechanisms are involved in the uptake of PS- MVs.

6.4 Limitations

A major flaw in the execution of this study was the anticoagulant used when collecting blood samples during the exercise trials. As MV and platelet poor plasma samples induced coagulation upon addition to assays, it can be assumed that the sodium citrate treatment of blood samples failed to prevent activation of platelets and coagulation factors. Sodium citrate is a chelating agent that interacts with calcium ions, preventing activation of platelets. It is probable that the sodium citrate was prepared using PBS rather than water, which would effectively deactivate the sodium citrate by overwhelming the chelating ability of the anticoagulant. Because calcium is involved in platelet activation, the deactivated anticoagulant would result in the activated platelets shedding MVs, inflating the concentration of MVs in the samples. Further, these PMVs would presumably have different properties than exercise-derived MVs and may account for the reduction in proliferation and wound healing seen in the functional assays. An alternate explanation for the sample coagulation is that the initial blood samples were not centrifuged at a high enough speed to completely remove platelets from the samples. Although a standardized protocol from the Society on Thrombosis and Haemostasis was used, reported centrifugation speeds for similar experiments are much higher, ranging from 3,200 x g to 13,000 x g.[10,34] Because of the procoagulant nature of the samples, the supernatant control had to be adjusted. The appropriate control would have been the plasma resulting from the initial centrifugation of MV samples, as this control would indicate whether other plasma constituents, such as lipids, proteins or exosomes, are responsible for any effects seen through the assays. However, as addition of plasma caused coagulation, it was not possible to use this treatment, and the PBS supernatant after washing MV samples was used instead.

Due to the coagulation issues, a third blockade study using annexin V, which binds PS and thus blocks PS interactions with other receptors, was not possible. Although washing the MV samples with PBS was sufficient to prevent coagulation, MV samples with annexin V caused coagulation because annexin V binding of PS is calcium dependent and the binding buffer contained calcium, which induces coagulation by activation of platelets and production of thrombin from prothrombin. This adds evidence to the theory that not all platelets and platelet fragments were removed with the relatively low speed centrifugation.

A major limitation to this study was the lack of laminar flow conditions to simulate the flow of blood within vessels, which is important for maintaining proper health and function of endothelial cells. Without flow conditions, it is unclear whether the results from this study, and others conducted under static conditions, can be translated to *in vivo* conditions. Without flow conditions, MVs can settle onto the endothelial cell monolayer and have a prolonged period to interact with the cells. However, under flow conditions, it is unclear whether MVs would be in contact with the endothelium long enough to allow uptake. Although implementation of a laminar flow chamber was considered for this project, there was not enough time to get one built. As well, while using a flow chamber would have allowed for conditions more similar to physiological conditions, few functional MV experiments have been conducted using flow chambers. As such, using a flow chamber would have made the results of the current study difficult to compare to relevant literature.

Another important element missing from this study is the quantitation of MVs. Without analysis of the samples, it cannot be concluded whether MV concentrations increased with exercise, or if MVs were present at all. It was planned that samples would be sent to Brunel University or the University of British Columbia Okanagan to be analyzed by flow cytometry. However, due to timing and logistical problems this never happened and samples were not sent elsewhere for analysis. Without a flow cytometer at Thompson Rivers University, quantification of MVs is difficult. However, MV concentrations could potentially be measured by electron microscopy, although this measure is fairly inaccurate and would only produce a rough estimate. Enzyme-linked immunosorbent assay could also be used to quantify MVs, but this would require an assumption of which proteins are present on the membrane of MVs. As exercise-derived MVs have yet to be characterized, assuming surface markers could result in an underestimation of MV

concentrations. However, both measures can be done with the resources available at Thompson Rivers University and would add value to future experiments.

7 CONCLUSIONS AND FUTURE WORK

The results of this study call into question the beneficial nature of MVs. While MVs have been associated with adaptive effects on the endothelium, they have also been implicated in disease states and coagulation. However, meaningful conclusions cannot be drawn from this research as results were not consistent between assays and contamination may have been responsible for some of the significant results. It is reasonable to conclude that the experiments should be repeated to determine whether the assays were working as expected. New samples should be obtained before repetition of assays, and anticoagulants should be tested before use. Quantification of MV samples would also add value as it is important to both verify the presence of MVs and to indicate whether effects are the result of increased MV concentrations or whether exercise MVs are inherently different from resting MVs. An important next step to understanding the function of exercise-derived MVs is characterization of the protein and mRNA content. This would supplement blockade assays by giving direct evidence of potential targets, rather than choosing targets based on studies that involve PMVs derived from other modes of activation.

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9 APPENDIX

9.1 Ethics approval certificate

THOMPSON RIVERS UNIVERSITY

May 11, 2016

Dr. Mark Rakobowchuk
Faculty of Science
Thompson Rivers University

File Number: 101200
Approval Date: May 11, 2016
Expiry Date: May 10, 2017

Dear Dr. Mark Rakobowchuk,

The Research Ethics Board has reviewed your application titled 'Understanding the role of microvesicles generated by exercise in healthy humans'. Your application has been approved. You may begin the proposed research. This REB approval, dated May 11, 2016, is valid for one year less a day: May 10, 2017.

Throughout the duration of this REB approval, all requests for modifications, renewals and serious adverse event reports are submitted via the Research Portal. To continue your proposed research beyond May 10, 2017, you must submit a Renewal Form before May 10, 2017. If your research ends before May 10, 2017, please submit a Final Report Form to close out REB approval monitoring efforts.

If you have any questions about the REB review & approval process, please contact the Research Ethics Office via 250.852.7122. If you encounter any issues when working in the Research Portal, please contact the Research Office at 250.371.5586.

Sincerely,

Andrew Fergus
Chair, Research Ethics Board